Cellular Mechanisms of L-arginine Induced Experimental Acute Pancreatitis

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Abbreviations

\[^{\text{Ca}^{2+}}\]_i intracellular calcium
Ca^{2+} free calcium
L-arg L-arginine
L-orn L-ornithine
Gly glycine
NO nitric oxide
NOS nitric oxide synthase
CaSR calcium sensing receptor
GPRC6a G-protein coupled receptor family C group 6 member A
GPCR G-protein coupled receptor
CCK cholecystokinin
DCF 2',7'-dichlorofluorescein
AP acute pancreatitis
ERCP endoscopic retrograde cholangiopancreatography
SIRS systemic inflammatory response syndrome
ARDS adult respiratory distress syndrome
CT computed tomography
IP\(_3\) inositol triphosphate
IP\(_3\)R inositol triphosphate receptor
ER endoplasmic reticulum
SERCA sarcoendoplasmic reticulum calcium ATPase
PMCA plasma membrane Ca\(^{2+}\) ATPase
ROS reactive oxygen species
RNS reactive nitrogen species
cGMP cyclic guanosine monophosphate
ONOO\(^-\) peroxynitrite
Abstract
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**Cellular Mechanisms of L-arginine Induced Experimental Acute Pancreatitis.**

**Introduction**

Impairment of cytosolic calcium ([Ca$^{2+}$]$_i$) signaling and in particular calcium overload has emerged as a possible unifying mechanism for precipitating acute pancreatitis (AP).

In the L-arginine (L-arg) experimental model of AP, nitric oxide (NO) has been implicated however the disease progression is largely unaffected by nitric oxide synthase (NOS) inhibitors (8). Additionally, L-ornithine (L-orn), a NOS-independent metabolite of L-arg, has been shown to be potent at inducing AP (28). Both L-arg and L-orn activate calcium-sensing like receptors (CaSR) (31) such as the GPRC6a which may be responsible for initiating the [Ca$^{2+}$]$_i$ overload.

The aim of this study is to investigate the effects of L-arg and L-orn on pancreatic acinar cells that maybe linked to the pathophysiology of AP. Furthermore to provide an alternative theory to the NO mediated ones, in particular that L-arg induces toxic changes in [Ca$^{2+}$]$_i$ via a GPRC6a like receptor.

**Methods**

Whole pancreata were harvested from male Sprague Dawley rats. Pancreatic acinar cells were isolated by collagenase digestion. [Ca$^{2+}$]$_i$ was measured using fura-2 imaging, and cell viability assessed using physiological CCK. Oxidative stress was measured using dichlorofluorescein (DCF) and cell death was quantified using trypan blue exclusion.

**Results**

*L-arg* and *L-orn* (100mM) induced spike-like, reversible increases in [Ca$^{2+}$]$_i$ in 46% and 74% of cells and Ca$^{2+}$ overload in 11% and 26% respectively. At 500 mM both induced Ca$^{2+}$ overload in all cells however this was also seen with the osmotic control, mannitol. Isosmotic L-arg and L-orn (100mM) induced only reversible increases in [Ca$^{2+}$].

Neither L-arg nor L-orn had significant effects on CCK-evoked [Ca$^{2+}$]$_i$ oscillations. Both L-arg and L-orn induced significant oxidative stress responses (22% and 37% of a maximum response seen with 3mM H$_2$O$_2$, respectively). Both L-arg and L-orn caused cell death in 76% +/- 4 and 89% +/- 7 at 3hours respectively, compared to 35% +/- 4 and 40% +/- 3 with controls (Hepes, Glycine).

**Conclusion**

The data suggests that the L-arg and L-orn causes significant increase in oxidative stress and cell death. The data suggests that although changes in [Ca$^{2+}$]$_i$ were induced by both L-arg and L-orn the large concentrations used experimentally are likely to induce significant osmotic effects.
DECLARATION

This MD thesis incorporates a series of experiments on oxidative cell injury carried out by Navdeep Gill (yr. 3 medical student, University Of Manchester) under my supervision, and formed part of his formal assessment for his BSc.

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Clinical relevance
In the western world the incidence of acute pancreatitis (AP) continues to grow. Coupled with the lack of therapeutic options this poses a significant global concern. AP has been defined as “an acute inflammatory process of the pancreas that frequently involves peri-pancreatic tissues and/or remote organ systems” (1). Over the past three decades there has been a great deal of successful work in identifying the causative agents and their mechanism of injury as well as the pathophysiology of disease progression. This mechanism includes: inflammation, acinar cell injury and cell necrosis/death. However what continues to cause controversy is the cellular events that trigger this initially local inflammatory response that has the potential to induce a catastrophic systemic inflammatory response syndrome (SIRS) with multiple organ involvement.

Large-scale cohort studies over the last decade have revealed an increased incidence in first attack of acute pancreatitis in western countries. Epidemiological data suggest the incidence to range between 10- 45 per 100,000 (2), with England being on the lower end of the scale and the United States on the upper. The incidence increases with age with a greater proportion of males than females in all studies (2). The multifactorial aetiology of AP is most likely to account for this geographical variation. Choledocholithiasis (e.g. gallstones) and alcohol abuse are implicated in most cases, accounting for 65-80% of all cases (3, 4). Other factors including: iatrogenic (ERCP and drug induced), hypertriglyceridaemia, autoimmune, traumatic and ischaemic are also well-recognised causes.

The pathobiology of the disease is characterised by: oedema, inflammation and varying degrees of parenchymal cell injury and death. This can vary from mild reversible pancreatic dysfunction to global organ necrosis and complete destruction of
the cellular infrastructure. Although the immune modulated inflammatory response is similar to that seen in other disease processes it is the characteristic auto-digestion of the gland that is the hallmark of the disease and responsible for the associated morbidity and mortality. The major pathological event is the inappropriate and premature activation of trypsin, which in turn activates a variety of injurious pancreatic enzymes, stored as zymogens. Enzymic injury coupled with a local immune response explains the intra-pancreatic inflammation. The next phase is extra-pancreatic inflammation which is well recognised to provoke a cytokine mediated systemic inflammatory response syndrome (SIRS) and subsequent remote organ involvement, in particular acute respiratory distress syndrome (ARDS) and acute renal failure.

Normal physiological exocytosis of pancreatic enzymes occurs as a result of an increase in cytosolic calcium ([Ca^{2+}]_i) but it is the initiation and progression of this process in AP that remains controversial. Sustained increase in [Ca^{2+}]_i is recognised to induce both necrotic and apoptotic cell death (5) and therefore abnormalities in [Ca^{2+}]_i homeostasis are pertinent to this discussion and will be explored in depth. The mainstay of current theories is centred on oxidative and nitrosative cell injury in particular by nitric oxide (NO). However a great deal of discrepancy exists within the literature as to whether NO has detrimental (6, 7) or beneficial effects in AP (8). This highlights the need for further exploration of these theories and to consider alternative ones.
Clinical diagnosis

It is generally accepted that a clinical diagnosis of AP can be made on serum levels of amylase and/or lipase. The levels of both these enzymes rise within hours of AP and will peak about 24hrs after onset of the disease. Serum lipase will remain elevated for longer than amylase but severity of disease does not correlate with the serum concentrations of either. Three times the upper limit of normal serum values for lipase/amylose is considered diagnostic. In 2006 an international gastroenterology symposium (9) suggested the diagnoses of AP requires two of the following three features; 1) serum amylase and/or lipase ≥ three times the upper limit of normal, 2) abdominal pain characteristic of AP and 3) radiological (CT) confirmation of: early pancreatitis with peri-pancreatic oedema or established organ necrosis, which is seen as poorly enhancing segments on contrast CT.

Clinical severity of early AP is determined by physiological derangement with remote organ dysfunction and later with pancreatic complications. Numerous physiological scores have been described that help quantify the severity of disease which can be related to prognosis. Early predictors of severity (first 48hrs) include: Ranson’s score (3), APACHE-II and The Glasgow Prognostic Criteria for severe acute pancreatitis (11). The Glasgow, or Imrie score (11), is widely used in the U.K as a useful prognostic scoring system for early acute pancreatitis. A score of three or more suggests severe AP, warranting patient care to be escalated to a high dependency or intensive care environment. The scoring indices include: arterial PaO2 <8kPa, age >55 years old, white cell count >15x10⁹/L, serum calcium <2mmol/L, serum urea >16mmol/L, serum lactate dehydrogenase>600iu/L, AST>200iu/L, serum albumin <32g/L and a blood glucose >10mmol/L.
Early AP can be limited to mild oedematous inflammation or can progress to organ necrosis. Secondary complications include the development of sterile or infected pancreatic pseudocyst. Necrosis is defined as non-enhancing segments on contrast CT greater than 3cm or greater than 30% of pancreas (4). Despite the advances in understanding the aetiology of the disease, its diagnosis and severity scoring, management of the condition is however supportive at best. The aim is to monitor closely and correct deviations in physiological parameters to minimise organ dysfunction. Currently there is no definitive treatment for AP. It is for this reason that comprehensive understanding of the initiating and early cellular triggering events of the disease process is crucial, however still lacking.

**Pancreatic acinar cell physiology**

Histological examination of the pancreas reveals essentially two types of parenchymal tissue fig. 1 (12). The lighter staining islet cells contribute to the endocrine function through insulin, glucagon and somatostatin. The peripherally orientated clusters, the acinar cells (acini), serve the exocrine function of the gland by producing the digestive enzymes: amylase, trypsin, lipase and chymotrypsin. The enzymic “pancreatic juice” is drained via pancreatic ducts into the main pancreatic duct. This duct then perforates the second part of the duodenum to introduce the pancreatic secretions to the intestinal contents.

Inappropriate activation of the proteolytic enzymes, stored in zymogens, leads to the characteristic autodigestion of the gland and is the hallmark of the disease.
Fig. 1. A diagramatic representation of the pancreas (12) showing: a) The anatomical association of the pancreas, 2nd part of the duodenum and drainage of the pancreatic and common bile ducts, b) clusters of pancreatic acini draining to the main pancreatic duct, c) the apical orientation of acinar cells containing zymogens and d) the lighter staining islet cells with perinsular acini.

It is well recognised that a rise in $[\text{Ca}^{2+}]_i$ precedes the normal exocytosis of digestive enzymes but a persistently raised concentration in $[\text{Ca}^{2+}]_i$ is known to be cytotoxic (5). Secretagogues such as acetylcholine and CCK generate characteristic, oscillatory changes in $[\text{Ca}^{2+}]_i$ which are associated with exocrine secretion (13). These agonists bind to specific G protein-coupled receptors (GPCR) which undergo a conformational change and subsequent production of secondary messengers, such as inositol 1,4,5 triphosphate (IP3) (14).

IP3 binds to specific receptors (IP3R) on the endoplasmic reticulum, the main store of $[\text{Ca}^{2+}]_i$. Stimulation of the IP3R on the ER results in oscillatory rises in cytosolic calcium in the apical pole of the acinar cell. These apically confined $\text{Ca}^{2+}$ oscillations have been linked to the exocytosis of zymogens into the pancreatic duct and duodenum (14). In pathological conditions such as reflux of bile acids or exposure to excessive ethanol metabolites, these $\text{Ca}^{2+}$ oscillations are thought to fuse into a
sustained irreversible rise in \([\text{Ca}^{2+}]_i\) leading to \(\text{Ca}^{2+}\) overload (13). This \([\text{Ca}^{2+}]_i\) overload results in necrotic cell death, which is the hallmark of AP.

Even in a resting state the ER leaks calcium into the cytosol this is countered by active uptake by the sarco/endoplasmic reticulum calcium ATPase (SERCA) pumps and by the plasma membrane calcium ATPase (PMCA) which pumps calcium out of the cell. This has been confirmed by an elevation in \([\text{Ca}^{2+}]_i\), as a result of thapsigargin inhibition of the SERCA pump (5). Numerous studies using calcium chelators have described the wide-ranging effects of a sustained rise in cytosolic calcium (15). These include mitochondrial dysfunction, premature activation of trypsinogen, cytokine expression, breach of plasma membrane and cell necrosis (16) that are all pathophysiological processes associated with AP. Cellular calcium homeostasis is clearly vital for cell viability and understanding this dynamic mechanism is important in AP.

The role of the GPCR is clearly an important component of normal acinar cell physiology and \(\text{Ca}^{2+}\) signalling. Of note is the calcium-sensing receptor (CaSR), a cell surface, G-protein-coupled receptor that senses minute changes in the extracellular (or plasma) \(\text{Ca}^{2+}\) concentration that couples to \([\text{Ca}^{2+}]_i\) signalling. It was first discovered in the parathyroid gland where it controls parathyroid hormone secretion and thus whole body \(\text{Ca}^{2+}\) homeostasis (15). Subsequently the CaSR has since been found to be expressed in many other tissues, such as kidney (19), bone (20) and intestine (18), where it has a similar role by controlling \(\text{Ca}^{2+}\) absorption/resorption. Expression of the CaSR has also been described in pancreatic ductal cells (17) where it is thought to reduce precipitation of ductal \(\text{Ca}^{2+}\) stones. A high concentration of \(\text{Ca}^{2+}\) in the secreted pancreatic fluid activates a CaSR to stimulate \(\text{HCO}_3^-\) secretion causing water to follow, reducing the lithogenic potential of the ductal fluid (17).
This receptor has also been shown to be activated by aromatic L-amino acids such as L-orn, L-lysine and L-arg (18). Amino acid activation of the CaSR has been demonstrated to induce a rise in $[\text{Ca}^{2+}]_{i}$ in human embryonic kidney cells (19). In addition to the CaSR another GPCR has been shown to be activated by L-amino acids, the GPRC6a (19). The role of GPRC6a in pancreatic acini has not previously been described however its presence has been shown in mice pancreatic β-cells and has a role in the recently described osteocalcin–insulin pathway (20). This pathway plays an important part in activation of osteoblasts and therefore bone and calcium homeostasis.

The role of these receptors, if any, has not previously been described in AP but clearly need to be explored and will be done in more depth in this body of work.

*Aetiology of AP*

AP has been associated with a number of aetiological factors. Many theories have emerged suggesting that various factors (hyperstimulation, bile salts, fatty acid ethyl esters) are linked to a disruption in the calcium regulating mechanism within the cell (13). Alcohol abuse is one of the main causes for AP however there is controversy surrounding the patho-physiology and in particular its impact on calcium regulation and the activation of intracellular enzymes. Many studies have reported that ethanol alone causes little disruption in acinar cell calcium signalling however its fatty acid metabolites have a profound effect (21). Both ethanol abuse and hypertriglyceridaemia lead to high levels of circulating fatty acid ethyl esters (FAEEs) which are recognised to both having an effect on IP3R calcium release and inhibiting mitochondrial respiration and ATP production (21). This in turn impairs SERCA and PMCA function leading to impairment in calcium clearance. Infusion of bile acids into the
pancreatic duct has also been shown to induce: calcium overload, mitochondrial depolarisation, ATP depletion and cellular necrosis (5).

Experimental stimulation of isolated acinar cells by physiological concentrations of CCK evokes characteristic spike-like changes in calcium (13). Interestingly higher concentrations of CCK induce a sustained rise in $[\text{Ca}^2+]_i$ with subsequent intracellular activation of trypsinogen (5,13). Furthermore administration of supra-physiological CCK is an established experimental mode of inducing AP (22).

The literature clearly demonstrates an in depth understanding of: normal pancreas physiology, the aetiology of AP, the progression of the disease and the systemic consequences. Despite this, however, there has been relatively little progression in disease modulating therapy. A possible explanation for this is that although disease detection is efficient clinically, from a physiological perspective the inflammatory process is already well established by the time the disease has manifested symptomatically. Efforts to improve our understanding of the earliest physiological processes i.e. cellular triggers of AP are key. To enable this it is crucial that an appropriate experimental model for AP is available and can easily be replicated to provide a focus for the necessary physiological analysis.

Numerous experimental models for replicating AP in the laboratory have been described. The L-arg. model is pertinent to this body of work and was compared to other widely used models.
Experimental Models for Pancreatitis

To validate theories on human disease progression it is useful to test them on an animal model that reflects the pathophysiological process seen in humans. Several experimental animal models of acute pancreatitis have been developed that essentially mimic many of the pathological changes that occur during the disease, such as pancreatic and end-organ injury (e.g. lung), elevated serum amylase, lipase, and inflammatory cytokines. These models include:

*Caerulein hyperstimulation*

High dose intravenous injection of the CCK analogue, caerulein, induces a hypersecretion of pancreatic enzymes. This has been seen to precipitate auto-digestion of the gland and therefore AP (23). Pancreatic interstitial oedema was seen one hour following IV administration. There has been success with this model in rats, mice, dogs, rabbits, pigs and Syrian hamsters (23).

The administration and monitoring of caerulein levels is a relatively simple procedure because of the intravenous mode used. This model has been shown to be particularly effective at representing extra-pancreatic complications of the disease such as: pulmonary pathology that develops as a result of ARDS, translocation of gut bacteria into the pancreas and blood. Additionally, once the dose of caerulein is discontinued, there is potential to investigate the recovery process of damaged tissue (23).
The caerulein model is considered useful in describing the early stages of acute pancreatitis (22). However, the major morphology in the model is that of oedematous pancreatitis. The difficulty in using this model to produce a severe, necrotising form of AP limits it use in translational work as it is the severe stages of the disease that contribute to the high morbidity and mortality rates in humans.

*Choline-deficient ethionine-supplemented diet (CDE diet)*

A method of inducing severe acute pancreatitis was described in 1975 that was based on controlling diet (24). Mice put on a choline-deficient ethionine-supplemented diet develop haemorrhagic pancreatitis with massive fat necrosis, although the diet can be modified to produce varying degrees of severity due to the synergism between choline and ethionine. The mechanism behind the CDE-diet is not fully understood, but it does have several features that make it useful in the study of pancreatitis. Particularly the fact it induces homogenously distributed inflammatory lesions and pancreatic necrosis. However there is wide-scale haemorrhagic destruction of the gland as well as necrosis, which result in a high mortality rate. It is comparatively cheap, simple, and the least invasive of all the models. Varying the degree of choline deficiency and ethionine supplementation allows some modulation of the associated mortality rate (24).

The CDE diet is considered a useful model for the middle stages of pancreatitis i.e. progression of necrosis (22). A huge limitation of the diet model, however, is that it is restricted to female mice; in other words, it is species and sex specific to a small animal, which makes this restrictive. The CDE diet also has direct effects on other
organs, namely the liver and brain. Thus it is non-specific within the body, and an equivalent degree of necrosis to that seen in humans has a much higher mortality rate in the animals (24).

Retrograde ductal injection of bile acids

This model involves the surgical cannulation of the pancreatic duct to provide a direct access to the pancreas. Bile salts, such as sodium taurocholate, are infused via this route, and induce a severe, acute pancreatitis, characterised by oedema, necrosis and haemorrhage. By varying the pressure of the infusion or the concentration of the agent, it is possible to control the severity of the disease. This mode of experimental AP has been successfully carried out in rats, rabbits, dogs and pigs (22). Bile duct infusion is an effective and reproducible method of creating a severe and rapidly evolving acute haemorrhagic pancreatitis with multiple organ failure, and it also acts as a useful model for studying fatty necrosis, pancreatic abscesses, and pseudocysts. There are technical issues, however, in terms of controlling the pressure of the infusion. Even normal saline will induce pancreatitis if infused at a high enough pressure, therefore tight control and monitoring of these pressures is necessary. This adds to an already technically demanding procedure. There is good flexibility in terms of the substances that can be infused, and allows the study of a variety of aetiological agents. Caerulein, for example, has been combined with glycodeoxycholic acid in the Boston model of acute pancreatitis (22).
Pancreatic stimulation in the presence of ductal obstruction (e.g. duodenal closed loop, duct ligation)-

From a clinical perspective a model of pancreatitis was needed to try and mimic the method by which gallstones lead to AP. This formed the basis of this particular model, which involves obstruction of the bile ducts, either by ligation of the common biliopancreatic duct, or by inserting a balloon-tipped catheter into it (22). It is thought that by blocking the ducts, the resulting bile reflux leads to intra-pancreatic digestive enzyme activation. This activity leads to rapidly developing and extensive necrosis, associated with a high mortality. This model has been successfully carried out on rats, rabbits and opossums (22). There is significant variation, however, in the severity of pancreatitis observed in the different species, thus making it difficult to assemble a standard model. Additionally, the procedure is expensive, highly invasive, technically demanding and therefore difficult to reproduce consistently as well as proving difficult to provide an adequate control for. Many of the symptoms are also more closely akin to chronic pancreatitis than acute (22).
**L-arg induced Acute Pancreatitis**

L-arg is a 4-carbon aliphatic straight chain amino acid. It is classified as a conditionally essential amino acid. Its distal end contains a complex guanidinium group that imparts a positive charge in most pH environments. It is synthesized from citrulline by the action of the cytosolic enzymes arginosuccinate synthesis and lyase (ASS, ASL). L-arg has been used extensively in cardiovascular research as an NO donor and much of its effects in vitro have been attributed to this metabolite.

Miznuma et al first described an important experimental use for this amino acid in 1984 (18), where it was shown that intra-peritoneal injection of high dose L-arg induced pathological and histological changes consistent with acute pancreatitis. This, relatively, non-invasive model has been reproduced and explored extensively over the past decade and is widely accepted as an experimental animal model for reproducing an acute necrotising pancreatitis that shares features of disease progression seen in humans.

The Miznuma et al model involved a single IP injection of 500mg/100g body mass of rat (bm). Raised serum lipase levels confirmed diagnosis of AP and this was further supported by micro- and macroscopic examination of the pancreas. Selective destruction of acini was seen as early as 24 hours with sparing of the Islets of Langerhans and adjacent acini. This periinsular sparing of acini is particularly interesting and has been further explored by Hegyi et al and have shown this to be abolished in rats who had streptozocin evoked diabetes (25).

Other histological features include peri-pancreatic oedema and fat necrosis. Early microscopic changes included ER disruption, vacuolisation of the cytoplasm and focal necrosis as well as leukocyte and fibroblast infiltration to mark the local inflammatory
process. These changes have been seen by other groups who have successfully reproduced this model (18, 25, 26, 27). The dose has been varied from <200mg to >500 mg / 100g bm and, in summary, doses less than 200mg/100g were unlikely to produce the disease characteristics and more than 500mg in one dose killed the animals. Hegyi et al (25) reported these changes as well as markers of a systemic inflammatory response with two IP injections of 250mg/100g given an hour apart. The study reported a rise in tumour necrosis factor-α (TNF-α), interleukin-6 and interleukin-1 within 24 hours of administration. This model has also been successfully reproduced in the mouse model using 400mg/100mg bm (27) where diagnosis of AP was confirmed by raised serum amylase which shared the time scale seen in humans, i.e. raised within 24-48 hrs, peak at 72 hrs and normalising in 96-120 hrs.

These experimental models serve an important purpose however many of these models are either too invasive (e.g. ductal obstruction) or they induce only mild forms of the disease (e.g. caerulein hyperstimulation.) Another important consideration is how readily reproducible the model is, for example, the CDE diet which produces a non-homegenous pancreatic inflammation. The L-arg induces a severe acute pancreatitis model, which is relatively non-invasive, easily reproducible, causes dose-dependent acinar cell necrosis, induces a systemic inflammatory response and the time scale of disease progression is similar to that seen in humans. This model can be used as a basis to improve our understanding of the cellular mechanisms that initiate and propagate the disease in this model.

The cellular processes and initiating mechanism for L-arg induced AP are not comprehensively understood. L-arg (fig 2) is metabolised by nitric oxide synthase
(NOS) to produce l-citrulline and NO. A NO-independent metabolism pathway also exists (fig 2) where L-arg is hydrolysed by arginase to L-orn and urea. L-orn has recently been shown to induce a more severe AP in rodents (28). This body of work challenges the dogma that exists that NO is the key mediator in cell injury in AP and will be discussed in more depth.

Figure 2. The metabolism of L-arg. There are two potential pathways, a NOS dependent one leading to the production of L-citrulline and NO. The other is arginase dependent and produces L-orn and also NO independent. The diagram also shows the potential effects of excess NO in the formation of toxic free radicals. L-orn forms an important precursor in cell division however it is has recently been shown to induce a severe AP in rats (28), the mechanism for this is not currently known.

NO has been linked to a multitude of essential processes including: modulation of growth hormone release, reduction of healing time in both bone and tissue, increase muscle mass, improve insulin sensitivity, and increase cardiovascular circulation.
Most of the current theories in this model of AP are centred on pancreatic injury being induced by nitrosative and oxygen free radicals (25).

However, several lines of evidence, using antioxidants, NOS inhibitors and NO donors (nitroprusside), suggest that these events are unlikely to be the trigger for L-arg-induced pancreatic damage (25, 28).

In physiological conditions, molecular oxygen exists in many states of excitation and oxidation, collectively known as reactive oxygen species (ROS). Reactive nitrogen species (RNS) refers to the different states and metabolites of NO. Theories regarding the mechanism of L-arg and L-orn induced AP is that they lead to the generation of oxygen free radicals and thus have their toxic effects via oxidative stress. Oxidative stress can be measured in pancreatic acinar cells by monitoring malondialdehyde (MDA) levels and dichlorofluorescein (DCF), which will be discussed in detail later. MDA is formed by the degradation of polyunsaturated lipids by ROS and is thus used as a biomarker for oxidative stress. MDA was recently used to investigate the pathological role of NO and found to be significantly raised 24 hours after high dose intra-peritoneal L-arg injection in rats and peaked at 48 hours (25). Further investigation has shown that preventing the generation of ROS caused a reduction in the severity of L-arg induced pancreatitis, therefore highlighting the role of L-arg, and possibly NO, in oxidative stress in AP. Dobosz et al (29) investigated this further by using a different model to delineate NO’s role in interacting with reactive oxygen species. They used sodium-taurocholate injection to induce AP and concluded that NO mediated oxidative stress was partly caused by peroxynitrite (ONOO’), which is formed by the reaction of NO with superoxide (O₂¬). A great deal of work has been done to ascertain both the physiological role of RNS and ROS as well as their
contribution to various disease processes in particular acute pancreatitis. The role of NO remains under debate, though having been implicated as pathological in AP (6,7), numerous studies have also shown its beneficial effects (8). Several lines of evidence, using antioxidants, NOS inhibitors (such as L-NAME) suggest that these events are unlikely to be the trigger for L-arg-induced pancreatic damage (25, 28).

NO is produced, in vitro, by the action of the enzyme nitric oxide synthase (NOS) and exerts it effects by the cyclic GMP pathway. The source of NO in the vascular endothelium, and to lesser extent acini, produces NO via eNOS (endothelial derived). The extra-pancreatic ganglia and intra-pancreatic nerve endings express nNOS (neuronal derived) and an inducible form is also present, iNOS. The source of this unclear but has been suggested as being mitochondrial in origin (30). Both eNOS and nNOS are calcium dependent and have a low NO output whereas iNOS appears to be insensitive to calcium and has a high NO output (30). The presence and activity of the enzymes is ascertained by measuring the pancreatic nitrite/nitrate directly. There are contrasting views with regards to the proportions of the enzymes present. DiMagno et al (31) proposed eNOS constitutes 30% in mouse acini whilst nNOS is less than 9%. Xu et al (32) described nNOS as being the most predominant in the rat model.

The majority of literature that supports a detrimental effect of NO suggests this occurs when it is produced in excess and in combination with ROS to form ONOO⁻. This form of oxidative injury through free radicals has been seen to cause rapid acinar cell destruction (33). The proposed mechanisms include injury to cell membrane/cytoskeleton, impaired intracellular protein, DNA damage, evoking lipid peroxidation and activation of NF-kB.

Paradoxically, there has been a significant body of work to support the beneficial effects of NO in the inflammatory process, in particular by enhancing pancreatic
blood flow. Dobosz et al (29) used laser doppler flow to measure microcirculation in pancreas, kidney, liver, lung and skeletal muscle. This was done following caerulein induced AP, in an animal, with both subsequent administration of L-arg (used as a NO donor) and NOS inhibitors. This study reported oedematous changes seen in milder clinical forms of AP through to pancreatic necrosis seen in severe cases. A significant reduction in microvascular blood flow (MBF) was seen universally in all test organs whilst in established AP. A significant and relatively fast improvement was seen following L-arg administration in the microcirculation. This was reinforced by the fact that NOS inhibition both further reduced pancreatic MBF and propagated severity of disease (29). NO was also shown to inhibit leukocyte activation and therefore reduce the inflammatory component of AP (34). NO related reduction of tissue injury through promoting MBF was also reported by other studies (35).

In addition to this another study revealed that inhibition of NO synthase (NOS) in L-arg pancreatitis failed to alter the histological appearance of the acinar cells (8). This suggests that NO has no significant role in L-arg induced AP.

The theory of nitrosative stress was challenged further when experiments showed that L-citrulline, the NO liberating metabolite, did not induce acute pancreatitis. However, the NOS-independent metabolite of L-arg, L-orn, did cause a severe, necrotising pancreatitis (28). L-orn is produced by arginase metabolism of L-arg (see fig 2).

Rakonczay et al (28) compared the metabolitess of L-arg in their ability to induce AP when injected intraperitoneally in wistar rats. These included: L-citrulline, NO (from sodium nitroprusside) and L-orn. Interestingly not only was L-orn the only one to induce AP but it produced a severe, necrotising AP at lower doses than L-arg (28). Additionally, levels of L-citrulline increased by approximately 3-fold following injection with L-arg; levels of L-orn, on the other hand, went up approximately 54-
fold in the same experiments. This suggests that L-arg brought about selective pancreatic acinar cell damage through L-orn rather than through formation of L-citrulline and NO. This body of work provides evidence to challenge the NO dogma that exists in AP theories. Understanding the mechanism of how L-orn may induce a cellular trigger for AP that is independent of NO is clearly necessary. The controversy surrounding NO suggests the need for exploring alternative theories to explain the early cellular events in L-arg induced AP.

Secretagogue induced changes in \([Ca^{2+}]_i\) are clearly a precipitating factor for normal zymogen exocytosis. However, stress induced calcium overload precipitates inappropriate enzymic activation and the hallmark of AP that is glandular autodigestion. This demonstrates a need to focus on this particular signalling pathway. There is currently no clear evidence linking NO and calcium signalling. In vitro oxidising agents have been seen to stimulate calcium oscillations, however these evoked responses did not result in enzyme secretion (13). Interestingly in salivary acinar cells NO has been shown to influence calcium release by increasing IP3 production (35). However numerous studies have shown little or no effects of NO on \([Ca^{2+}]_i\). In fact \(Ca^{2+}\) overload has been suggested to be a common early pathological trigger in AP regardless of NO.

In view of the lack of convincing and consistent evidence for role of NO in L-arg induced AP, it is reasonable to suggest an alternative mechanism that may involve \(Ca^{2+}\) overload.

As previously discussed the secretagogues CCK and ACh induce IP3 mediated changes in \([Ca^{2+}]_i\) through activation of GPCR’s. It was also noted that a particular G-protein complex exists that is activated by L-aromatic amino acids, the GPRC6a. GPRC6a was first described in the parathyroid gland where it controls parathyroid
hormone secretion and thus whole body Ca\(^{2+}\) homeostasis (37). Both L-arg and its NO independent metabolite, L-orn, have been shown to induce a severe AP in rodents as well as being recognised activators of the GPRC6a; a related putative amino acid sensing receptor. This transmembrane protein can induce changes in [Ca\(^{2+}\)]\(_i\) as a result of extracellular ligand binding through production of the intracellular messenger IP3. There is a lot of evidence within in the literature for the L-amino acid activation of GPRC6a (19,37,38). The table below shows calculated EC\(_{50}\) for the receptor (table 1)

<table>
<thead>
<tr>
<th>L-amino acid</th>
<th>EC(_{50}) (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-orn</td>
<td>63.6</td>
</tr>
<tr>
<td>L-lysine</td>
<td>135</td>
</tr>
<tr>
<td>L-arg</td>
<td>284</td>
</tr>
<tr>
<td>L-cysteine</td>
<td>356</td>
</tr>
<tr>
<td>L-alanine</td>
<td>486</td>
</tr>
<tr>
<td>Glycine</td>
<td>538</td>
</tr>
</tbody>
</table>

Table 1. EC\(_{50}\) values for L-amino acid activation of wild-type mouse GPRC6a (19).

There is, however, some discrepancy in the literature with regards to the potency of theses amino acids activating GPRC6a. Table 1 refers to data obtained from wild-type mouse GPRC6a assay. Similar work has also been carried out on cloned rat GPRC6a (table 2). Although both studies support the amino acid activation of GPRC6a they differ in their potency. Species variation may well account for this. It is also important to note the values for rat GPRC6a are on isolated, cloned receptors in an overexpressed system.
Whilst the presence of CaSR’s in pancreatic acini has not previously been described the fact that they can induce changes in cytosolic calcium as a result of extracellular L-amino acid binding suggests their potential role needs to be explored. In particular that L-arg may activate a calcium-sensing receptor leading to an irreversible Ca\(^{2+}\) overload response. This toxic calcium signal may induce its effect through premature, inappropriate activation of zymogens and consequent necrotic cell death.

Acute pancreatitis is clearly a global concern with an unacceptably high mortality rate as a result of a lack of definitive treatment. Animal models developed over the past two decades have enabled greater understanding of the pathological features and progression of the disease. However, many of these models are either too invasive (e.g. ductal obstruction), induce only mild forms of the disease (e.g. caerulein hyperstimulation), produce non-homogeneous pancreatic damage or are not reproducible (e.g. CDE diet). The L-arg and L-orn induced models, on the other hand,
are relatively non-invasive, easily reproducible, cause dose dependent acinar cell necrosis, induce a systemic inflammatory response and the time scale of disease progression is similar to that seen in humans. Understanding the cellular mechanisms that initiate and propagate disease in these models is crucial to furthering attempts in modulating the disease in humans. The dogma that exists is that in this model the pancreatic injury is induced by NO and oxygen free radicals. There is doubt about the role of NO, if any, and the controversy in the current literature suggests there is a need to consider alternative theories to explain the initiating cellular mechanism. In particular there is a need to clarify the effects of L-arg and L-orn on acinar cells and consider the possibility they may activate a calcium-sensing receptor leading to an irreversible Ca\(^{2+}\) overload response and a consequent necrotic cell death.
Aims of Current Study

The overarching aim of this study is to improve our understanding of the earliest cellular triggers to AP. L-arg causes AP in rodents that mimics many features of the disease in humans. The focus of this study is to explore the effects of L-arg on pancreatic acini and to identify a link between these and AP.

The gap in the literature demonstrates there is a need to understand the biomechanisms involved in triggering AP in the animal model. This serves not only to improve our understanding and application of the experimental model but also with the potential of improving our understanding of the disease in humans.

Currently the exact mechanism of how L-amino acid induces AP is unclear. The historic use of L-arg in cardiovascular medical research as NO donor combined with the well know cytotoxic capabilities of reactive oxygen and nitrogen species forms the basis of current theories. The current evidence also reveals inconsistencies in this theory and raises the possibility of alternative ones. Namely that it is well recognised that a disruption in intracellular signalling, in particular calcium related, has the potential to explain the earliest cellular processes that are seen in AP. As previously described one of the main theories explored in this study is that experimental L-arg induced AP is initiated by activation of a GPCR such as the calcium sensing like receptor (CaSR) or the GPRC6a. GPRC6a was first described in the parathyroid gland where it controls parathyroid hormone secretion and thus whole body Ca\textsuperscript{2+} homeostasis (37). Both L-arg and its NO independent metabolite, L-orn, have been shown to induce a severe AP in rodents as well as being recognised activators of the GPRC6a; a related putative amino acid sensing receptor. This transmembrane protein can induce changes in [Ca\textsuperscript{2+}]\textsubscript{i} as a result of extracellular ligand binding through production of the intracellular messenger IP3. The question that arises is, if these
aromatic amino acids act as ligands for this G-protein or other CaSRs in pancreatic acini, could this explain the early cellular events in AP?

To provide further information to test these hypotheses the following questions arise:

1) *What effects, if any, do L-arg and L-orn have on [Ca\(^{2+}\)]\(_i\)?*

   The natural starting point is to establish if these amino acids have any effects and if so whether these changes could help explain what is seen in the experimental model.

2) *Are these L-arg induced changes in calcium consistent with receptor-mediated responses?*

   If so the next step would be to identify if a CaSR or GPRC6a receptor is expressed.

3) *Compare the effects of L-arg and L-orn to the physiological calcium signals induced by secretagogues such as CCK.*

   The well-described G-protein receptor coupled response seen with CCK is a good model for comparison. As previously mentioned CCK, in excess, can induce AP through IP3 mediated calcium overload resulting in inappropriate enzymic activation (23). It activates specific G-protein receptors, which exist as subtypes CCK-1 and CCK-2 (also known as a and b types). These receptors can be modulated by agonists such as caerulein (used experimentally) and antagonists such as proglumide, used in the treatment of peptic ulcer disease. Aromatic L-amino acids are known to activate receptors such as GPRC6a however their effect, if any, on CCK receptors have not previously been described. In the experimental models acinar cells will not only be exposed to the reagents but also to circulating CCK. There is a potential interaction either
at the receptor level or with the $[\text{Ca}^{2+}]$, signal. It is important to establish if L-arg and L-orn have any impact on the physiological CCK evoked response.

4) *What effects do L-arg and L-orn have on intracellular oxidative stress?*

As oxidative injury has been a focus within the literature it is important to establish if these amino acids induce any oxidative stress in acinar cells at the concentrations used experimentally.

5) *Are L-arg and L-orn directly cytotoxic and at what concentrations?*

Establishing these amino acid’s ability to cause cell death is an important baseline in exploring this experimental model of AP.

6) *A comparison of the effects of L-orn with those of L-arg.*

To further explore the theories in the literature it is important to establish the impact of L-arg and more importantly L-orn on intracellular NO. L-arg has a well-established history as a NO donor. Another pathway exists which leads to the production L-orn and is independent of NO. This metabolite appears to be more potent at inducing AP experimentally and appears to be the more prevalent metabolite in L-arg induced AP (28). The effects of L-orn on intracellular NO are of particular interest.

Obtaining information about the acinar cellular processes seen as a result of exposure to L-arg at the concentrations used experimentally is key to help bridge the gap that exists in the literature.
Materials and Methods

Experimental Design

The key areas in designing experiments included:

1) **Isolation of rat pancreatic acinar cells**

The method used to isolate acinar cells from rat pancreata involved a whole organ collagenase digestion technique adapted from Bruce et al 2007 (39)

2) **Calcium imaging experiments**

The focus of this body of work was on intracellular calcium signalling in acinar cells. Cell selection is clearly necessary before obtaining reliable data. However cautiously the isolation procedure is done there are likely to be a small number of damaged or dead cells. An appropriate ratiometric calcium indicator dye such as fura-2 to create cell fluorescence was used to visualise the cells. Visual analysis revealed some of the damaged cells with regards to gross structural abnormalities and the resting calcium state. Functional analysis, however, is required to assess physiological viability. As the initial experiments are based on measuring changes in calcium a secretagogue such as CCK was used to assess cell function.

2.1 **The effects of L-arg and L-orn on resting calcium state in a viable acinar cell.**

The test reagents concentrations were based on the previously described L-arg (18,27) and L-orn (28) models for inducing pancreatitis. L-arg and L-orn were selected as the test reagents, glycine as a negative experimental control (28) and mannitol as an
appropriate osmotic control. A series of experiments were carried out on viable acinar cells loaded with Fura-2 ratiometric dye.

2.2 Effects on CCK oscillations.
Exocrine function in pancreas is modulated by secretagogues such as CCK. There is the potential that the effects seen experimentally with L-arg maybe as a result of interference of the normal physiological response to this peptide agonist. To test this theory L-arg and L-orn were added to cells displaying an established train of CCK-induced oscillations.

3) Oxidative stress experiments.
Oxygen free radicals have been incriminated in the patho-physiology of AP. To establish if L-arg and L-orn induce intracellular oxidative stress their responses were compared to those seen with a potent oxidising agent, hydrogen peroxide. Oxidative stress was measured using the highly fluorescent dichlorofluorescein which reacts with hydroperoxides.

4) Cell death studies.
The literature reveals that the cellular mechanisms involved in L-arg induced AP are unclear. One of the essential factors is to establish whether L-arg and L-orn are cytotoxic and at what concentration. A relatively simple way to achieve this is by a dye exclusion test to determine the number of viable cells present in a cell suspension. Trypan blue is excluded from cells with an intact membrane and therefore can be used to visualize dead cells.
5) NO studies.

The exact cellular mechanism of L-arg induced AP in not known and there is assumption made the effects are attributed to L-arg acting as a source of NO. Furthermore the NO independent metabolite, L-orn, is more potent at inducing AP and little is known about the action of this at a cellular level. Investigation of the changes in intracellular NO induced by both L-arg and L-orn was carried out using 4,5-diaminofluorescein diacetate (DAF-2 diacetate).

For the purpose of investigating the cellular processes that occur during L-arg induced experimental AP in rodents it was necessary to identify and measure the earliest intracellular changes within the pancreatic acini as a result of exposure to the named reagents. To enable this, clusters of pancreatic acini were isolated from healthy rats. The materials and methods used are as follows.
Methods

(All solutions used with their respective constituents are listed in table 3)

1) Isolation of pancreatic acinar cells

All animals used were male Sprague-Dawley rats between 200 and 300g. Animals were killed in accordance to Home Office Guidelines (schedule 1) by cervical dislocation. The peritoneal cavity was accessed via a midline incision and the pancreas was resected as a whole. The tissue was then injected with 10ml of cold collagenase solution (800 units of collagenase-P per gram of tissue [Sigma Type II], 0.12mg/ml soybean trypsin inhibitor [Sigma Aldrich] and 1% bovine serum albumin BSA [Fraction V, Sigma]) in HEPES-buffered physiological saline solution (HEPES-PSS, composition in mM: 137 Na⁺, 0.56 Mg²⁺, 1 HPO₄²⁻, 4.7 K⁺, 10 HEPES, 5.5 glucose and with a pH of 7.4).

Excess connective tissue and fat were excised and the pancreatic tissue was minced using scissors. The tissue was incubated in the collagenase solution at 37°C for 15 min in a shaking water bath. The supernatant fluid was aspirated and replaced with 15mls of fresh collagenase solution and incubated for a further 15 min. This process was repeated replacing the supernatant with 10mls of 5mM EDTA (in HEPES-PSS with 0.12 mg/ml solution of trypsin inhibitor; Sigma Aldrich). The sample was incubated for a further 10 min. Following this tissue was gently washed using the generic incubation solution (GIS-0.12mg/ml soybean trypsin inhibitor and 1% BSA in HEPES-PSS, Sigma Aldrich) to remove the EDTA. The tissue sample was triturated in GIS using progressively smaller pipette tips and then filtered through a nylon mesh to produce a suspension of pancreatic acinar cells. This was then layered onto 4% BSA solution (containing 0.12mg/ml of trypsin inhibitor, Sigma Aldrich.) and
centrifuged at 620g for two minutes. This process was repeated to remove further dead cells. The resulting pellet was re-suspended in GIS and stored on ice ready for use.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Constituents</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEPES-PSS</td>
<td>NaCl – 137mM</td>
</tr>
<tr>
<td><strong>HEPES physiological saline solution</strong></td>
<td>HEPES – 10mM</td>
</tr>
<tr>
<td></td>
<td>Na$_2$HPO$_4$ – 1mM</td>
</tr>
<tr>
<td></td>
<td>MgCl$_2$ – 0.56mM</td>
</tr>
<tr>
<td></td>
<td>Glucose – 5.5mM</td>
</tr>
<tr>
<td></td>
<td>CaCl$_2$ – 1.28mM</td>
</tr>
<tr>
<td></td>
<td>KCl – 4.7mM</td>
</tr>
<tr>
<td>Generic incubation solution (in HEPES-PSS)-GIS</td>
<td>Bovine serum albumin (BSA) – 1%.</td>
</tr>
<tr>
<td></td>
<td>Trypsin inhibitor – 0.12mg/ml, Type II-S from Soybean.</td>
</tr>
<tr>
<td>Collagenase (in generic incubation Sol.)</td>
<td>Collagenase – 0.1mg/ml, from <em>Clostridium histolyticum</em>, Roche</td>
</tr>
<tr>
<td>EDTA (in HEPES-PSS)</td>
<td>EDTA – 5mM</td>
</tr>
<tr>
<td></td>
<td>Trypsin inhibitor 0.12 mg/ml</td>
</tr>
<tr>
<td>4% BSA (in HEPES-PSS)</td>
<td>BSA – 4%</td>
</tr>
<tr>
<td></td>
<td>Trypsin inhibitor – 0.12 mg/ml</td>
</tr>
<tr>
<td>Fura-2 acetoxyethylene dye (in HEPES-PSS)</td>
<td>Fura 2. - 3-5 µM</td>
</tr>
</tbody>
</table>

*(Invitrogen Molecular Probes)*

*Table 3. Solutions used in the isolation process of rat pancreas acini. All materials were obtained from Sigma Aldrich and Calbiochem unless otherwise stated. All solutions were corrected to pH 7.4*
Methods;

2) Calcium imaging experiments

*Digital imaging of fura-2 fluorescence (measurement of intracellular [Ca\(^{2+}\)]\(_i\))*

The pancreatic acinar cell suspension was centrifuged at 2000 rpm and the subsequent pellet was incubated in 4µM fura-2 acetoxyethyl ester (fura-2 AM; TEF Labs/Stratech, Soham, UK) in HEPES-PSS for forty minutes at room temperature. A further de-esterification step is necessary to prevent dye leakage; this involved repeating the procedure but incubating in the GIS.

The loaded pancreatic acinar cells were allowed to adhere onto glass cover-slips and mounted in a gravity fed perfusion chamber at room temperature. The chamber inflow allowed up to six different solutions to be administered with automatic valves for rapid switching of solutions (Harvard Apparatus, Kent, UK.) A vacuum driven outflow maintained the dynamics of the perfusion.

Fluorescent cell images were acquired using either a Nikon Diaphot 300 microscope (equipped with an ORCA-ER CCD camera, Hamamatsu) or an inverted epifluorescence Nikon TE2000 microscope with a coolSNAP HQ interline progressive scan CCD camera (Roper Scientific Photometrics). A 40x oil immersion lens with a numerical aperture of 1.3 was used on both microscopes. Cell illumination was achieved with a xenon arc lamp (Nikon) and a Cairn optoscan monochromator (Cairn Research). Cells were excited at 340 nm and 380 nm and the emitted fluorescence was captured through a 400 nm diachroic mirror, with long-pass filter, onto the camera. The devices were co-ordinated with the image acquisition software, Metaflour (Molecular Devices) as well as recording the images. Background-subtracted images at both 340 nm and 380 nm fluorescence were captured with 5x5
binning at a rate of 0.2Hz and 50ms exposure. The fura-2 340/380 ratio represented the change in intracellular calcium. This information was represented both as images and the data was plotted against time.

Cell selection was based on the following criteria: clusters of approximately 3-6 cells, well defined cells with healthy, intact membranes and with the electron-dense secretory granules at the apical end of each cell aligned to the middle of the cluster, indicating a high degree of polarisation, individual signal saturation<4000 (levels of grey) and 340/380 ratio less than 1.

2.1 The effects of L-arg and L-orn on resting calcium state in a viable acinar cell

After the cells had been allowed to adhere and appropriate ones selected, the perfusion was activated. Initial perfusion was with phosphate free HEPES-PSS, to prevent solute precipitation. 100pm CCK was used to test cell viability by evoking real, “spike like”, calcium oscillations. If successful the CCK was then washed out with HEPES control until no further oscillations were seen and the next step of introducing the chosen reagent could be initiated.

The rationale behind choosing the concentrations of the reagents was based upon the description, in the literature, of the experimental models. The concentrations for L-arg used in the animal models were 500mg/100g body mass of rat (27) as a single intraperitoneal injection and 250mg/100g (27) as two separate injections an hour apart. As a maximum of 4mls can be injected in the animal at a time the concentrations used for a 300g rat ranged from 1.01M to 2.15 M. Dilution effects due to fluid sequestration from abdominal viscera as a result of infusion with a hyperosmolar solution must be taken into account as well as the effects of the fluid exudation induced by chemical peritonitis. Therefore the concentrations of reagents that the pancreas is exposed to
will be much less than these calculated values. The concentrations used in this study ranged from 10 mM to 500 mM to account for the maximum, feasible dilution effects occurring in vitro.

Rakonczay et al demonstrated another mode of inducing a severe AP using L-orn (28). This amino acid is a NO independent metabolite of L-arg and also known to activate GPRC6a and was therefore pertinent to this study. L-arg and L-orn were the selected test reagents in all experiments. The negative experimental control used was glycine (28) and therefore an appropriate amino acid control for this study.

Using high concentrations of reagents warranted the need for a control that was equal in osmolarity but did not enter the cell, an appropriate choice was mannitol.

Further experiments were carried out to investigate the effects of abolishing the osmolarity differences. This was done by using a HEPES- physiological saline solution where 100mmol of NaCl were replaced with L-arg to produce a reagent with an osmolarity and content comparable to the HEPES-PSS control solution the cells were incubated in. The control used in this experiment was a HEPES-PSS solution in which the NaCl was replaced with NMDG. This control was used to account for effects due to sodium depletion. Reducing the concentration of [Na$^+$] and [Cl$^-$] ions can have an impact on cell membrane potential and transporters however as the intracellular concentration of [Na] is 5 mM replacing only 100 of the 140 mM of NaCl should maintain the positive gradient allowing [Na] influx (Table 4 – test reagents.)
<table>
<thead>
<tr>
<th>Reagent</th>
<th>Details</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEPES-PSS phosphate free</td>
<td>Control solution</td>
<td>osmolarity- 0.274 osmol/kg</td>
</tr>
<tr>
<td>CCK</td>
<td>Cell viability control</td>
<td>100 pM</td>
</tr>
<tr>
<td>L-ARG</td>
<td>Grade &gt;98% (Sigma Aldrich)</td>
<td>10, 100, 200, 300, 500 mM</td>
</tr>
<tr>
<td>L-ORN</td>
<td>L-orn hydrochloride 99% (Sigma Life Science)</td>
<td>10, 100, 300, 500 mM</td>
</tr>
<tr>
<td>GLYCINE</td>
<td>Used clinically as negative control</td>
<td>10, 100, 200, 300, 500 mM</td>
</tr>
<tr>
<td>MANNITOL</td>
<td>Osmolarity equal negative control</td>
<td>10, 200, 500 mM</td>
</tr>
<tr>
<td>Isosmotic L-ARG</td>
<td>L-arg in HEPES-PSS, osmotically equal solution</td>
<td>100 mM L-arg and 37mM Na (osmolarity- 0.268 osmol/kg)</td>
</tr>
<tr>
<td>Isosomotic NMDG</td>
<td>NMDG in HEPES-PSS, control for removal of Na</td>
<td>100mM NMDG and 37mM Na (osmolarity- 0.293)</td>
</tr>
</tbody>
</table>

*Table 4. Experimental test and control reagents used for the described calcium signalling experiments carried out on isolated acinar cells.*
The data values obtained at each wavelength (340 and 380 nm) represent intracellular calcium and were recorded at 5 second intervals. A true oscillatory increase in $[\text{Ca}^{2+}]_i$ is represented by an increase in the 340 signal matched by a decrease in the 380 signal over the same time period (see fig 3). This results in a rise in the 340/380 ratio, these values were recorded and plotted against time. The highlighted area on figure 3 represents real changes in calcium induced by the test reagent.
Figure 3. Measuring Ca²⁺ changes in Fura 2-loaded acinar cells. a. Represents an actual image of a cluster of fura-2 loaded acinar cells displaying a change in fluorescence when excited at wavelengths 340nm and 380nm, b. is an actual tracing obtained with “Metafluor” showing the 380nm (solid lines) and 340nm (dotted lines) traces in response to physiological CCK and then the test reagent; 100mM L-arg (highlighted area.) This peak seen on the 340/380 ratio graph implied a “real” change in Ca²⁺.
2.2 Effects on CCK oscillations

These series of experiments required measuring fluorescence in fura-2 loaded acinar cells as previously described. Appropriate acinar cells were selected and allowed to adhere before commencing perfusion. These were then perfused for ten minutes with 50 pM CCK to induce the characteristic spike like changes in calcium.

![Figure 4. A typical oscillatory, spike-like calcium response seen induced by 50 pM CCK](image)

Once stable calcium oscillations were established, the test reagent solution containing 50 pM CCK was then added. Reagent solutions used were isosmotically corrected 100mM L-arg, L-orn and NMDG. Following the test period the reagent solution was replaced with one containing the control (50 pM CCK alone). This was done to establish any residual effects on CCK evoked responses.
3) Assessment of cell death – Trypan blue exclusion

L-arg and L-orn have been shown to induce AP in animal models. The exact mechanism for this is not fully understood. This series of experiments is intended to establish whether these L-amino acids are directly cytotoxic. Necrotic cell death was assessed using the DNA-binding histological dye, trypan blue.

A 0.4% trypan blue solution was initially made dissolving 0.4g of trypan blue granules (Sigma Aldrich) in 100 ml of phosphate buffering solution (Sigma Aldrich). The final trypan blue dye solution (TBS) required adding HEPES-PSS and 4% formaldehyde fixing solution (Sigma Aldrich.) A 1:1 acinar cell to TBS suspension was left to incubate for 3 minutes at room temperature.

A Neubauer haemocytometer (Haksley and Sons) was used to stage the cell suspension. Both chambers of the haemocytometer were covered with glass cover slips and 20µl of cell suspension were transferred to each chamber by means of an air displacement pipette. Visualisation of the cells was achieved using a standard light microscope with a 10x objective lens.

As previously described trypan blue stains cells in which the membrane has been breached allowing the dye to bind to the nucleus, highlighting dead or damaged cells. The outer 4 squares of the 9 square grid were used for counting cells (fig 5). This was done for both chambers in the haemocytometer. The number of dead cells were expressed as a percentage of the total number of cells and recorded. All data were compared to a time-matched control.
A diagrammatic representation of a haemocytometer highlighting the area used for counting cells. The cells in black represent dead cells and would be expressed as a percentage of the whole number of cells in the test field.

4) Oxidative stress experiments

Digital imaging of dichlorofluorescein fluorescence

As previously discussed oxygen free radicals feature in many of the current theories in AP. These series of experiments were aimed at establishing whether L-arg and L-orn induce significant cellular oxidative stress. Intracellular oxidative stress was assessed using the fluorescent dye dichlorofluorescein diacetate (DCFH-DA.) DCFH-DA is cleaved intracellularly by esterases to produces dichlorofluorescein (DCF). DCF is highly fluorescent on reacting with hydroperoxides and therefore an ideal
choice for measuring intracellular oxidative stress. The intensity of DCF fluorescence is proportional to the level of reactive oxygen species within the cell. In particular the cytotoxic species, peroxynitrite, can oxidise DCF directly (40).

Isolation and selection of pancreatic acini were carried out as described previously. Acinar cells were centrifuged and washed with HEPES-PSS twice before loading. The cells were incubated with 10μM H$_2$DCF-DA solution (Molecular Probes/Invitrogen, Paisley, UK) (in HEPES-PSS) for 30 minutes at room temperature. The loaded cells were re-suspended and kept on ice until needed.

Imaging was carried out using the same equipment and technique as described above for the calcium imaging experiments. However, the images were obtained using a fluorescein filter set. The excitation spectrum was at 480nm and the emitted fluorescence was at 530nm, which was separated using an FITC dichroic mirror. The cells were added to a perfusion chamber of HEPES - PSS and left to adhere to the cover slip for 2 minutes. Healthy cell clusters were selected for imaging based on the criteria outlined above as well as ensuring that cells that had abnormally high levels of fluorescence were excluded, as this indicated high resting levels of oxidative stress and probable cell death. The perfusion of HEPES was restarted and imaging at 480nm was commenced every 5 seconds with an exposure time of 50-100 msec. An initial control period of approximately 10 minutes was run using a perfusion of HEPES - PSS. After this, the reagent to be tested was added and the results recorded for 10 further minutes. As a positive control, 3mM H$_2$O$_2$ was added to produce a robust oxidative stress response. All imaging experiments were done at room temperature in a dark environment. All the reagents tested were isosmotically corrected to HEPES-PSS to eliminate the osmotic effects on the cell (previously described.)
5) Measuring changes in intracellular NO

Controversy exists around the role of NO in the earliest stage of AP. An important baseline is to establish the changes, if any, in intracellular NO induced by L-arg and L-orn.

Kojima and collaborators’ developed a successful nitric oxide indicator in form of 4,5-diaminofluorescein diacetate (DAF-2 diacetate) (47). A related dye, 4-amino-5-methylamino-2′,7′-difluorofluorescein (DAF-FM) has also been used to measure nitric oxide production in living cells and solutions (41).

Isolated acinar cells were incubated with 5µM DAF-FM solution (Molecular Probes/Invitrogen, Paisley, UK) (in HEPES-PS) for 40 minutes at 37°C (8). The loaded cells were then centrifuged, re-suspended and kept on ice until needed. The imaging technique was the same as that used in the DCF experiments. The cells were added to static perfusion of HEPES – PSS and left to adhere to the cover slip for 2 minutes. Healthy cell clusters were selected for imaging based on the criteria outlined above. The perfusion of HEPES - PSS was restarted and imaging at 480nm was commenced every 5 seconds with an exposure time of 50-100 msec. After a control period of approximately 5 minutes, the cell sample was perfused with an NO donor. Multiple NO donors were used to try and elicit a fluorescent response within each experiment, these included: sodium nitroprusside, SNAP and glyceryl trinitrate (GTN.) These acted as controls for a positive NO response and provided comparison for the test reagents L-arg and L-orn.
Results

Calcium imaging experiments

L-arg induced a robust increase in $[\text{Ca}^{2+}]_i$.

The data were interpreted using three different approaches:

1) *Categorisation by shape of the curve produced by plotting the 340/380 ratio vs. time.* L-arg and L-orn induced robust concentration dependent increases in $[\text{Ca}^{2+}]_i$. Some cells exhibited a spike-like oscillatory response whereas others exhibited a slow sustained increase in $[\text{Ca}^{2+}]_i$. These responses were highly heterogeneous and in order to quantify this heterogeneity, responses were categorised and the number of cells that exhibited each type of response were determined for each reagent (fig. 6). This form of categorisation has been adapted from Bruce et al 2007 (39).
Figure 6. Categorisation of responses (1st marker signifies CCK perfusion, 2nd marker signifies reagent perfusion, MMR-the mean maximum response, AUC-The area under the curve)

The initial stimulation of the cell with a physiological concentration of the agonist CCK enabled assessment of cell viability. An appropriate response with CCK was of oscillatory changes in cytosolic calcium as shown in fig 4, anything other than this was excluded as a non-viable cell cluster and therefore not used. Once cell viability was established CCK was stopped and the cell was allowed to return to a resting state before the reagent was commenced. The responses were then identified and categorised as shown in fig 6. It is important when interpreting this data to understand the implications of the shape of the response curve:

*Category A* represents a viable cell in which the reagent did not evoke any real changes in intracellular calcium.
Category B illustrates a viable cell that demonstrates a rapid increase in the 340/380 ratio in response to the reagent with an immediate return to the baseline. This change gives a “spike” like appearance to the curve. This implies that the reagent evokes a rapid rise in intracellular calcium with equally swift calcium clearance. This spike like response is reminiscent of a receptor mediated response in a cell with intact calcium clearance mechanism. This is the most similar to a physiological CCK type response.

Category C is a viable cell that produces a broad-based response. As shown in fig. 6 a category C response is also initially rapid with a sharp peak however the important difference between category C and B is the time scale for recovery. This could be as a result of a different mechanism of inducing the increase in cytosolic calcium and or impairment in the calcium clearance mechanism. It is important to note that although the return to the baseline is gradual it does occur suggesting that cell death has not occurred.

Category D is a viable cell in which the reagent has evoked a significant increase in cytosolic calcium, which continues to rise up to a plateau. The result is a cell with an irreversible increase in [Ca^{2+}]i. This high level of cytosolic calcium is likely to represent cell injury / cell death.

It is important to note the progression from category A to D represents an increase in severity from “spike-like” physiological responses (B) to “pathological” irreversible calcium overload (D).

At concentrations below 100mM L-arg did not evoke any changes in [Ca^{2+}]i (fig. 7). However, 50% of cells produced a “spike” like (type B response) at 100mM, increasing to 70% at 200mM. There is a significant change in the type of responses seen at concentrations higher than this. At 400 and 500mM the vast majority of
responses were of type C and D respectively. As previously discussed these responses suggest that either a different mechanism is involved in inducing the increase in [Ca^{2+}]_{i} or an impairment in the calcium clearance mechanism might have occurred.

There appears to be a threshold at 100 mM below which the cells do not respond to L-arg and then a narrow range in which there is an increasing proportion of cells responding in a physiological manner (i.e. type B response) before there is a marked change with an increasing percentage of cells displaying an irreversible increase in calcium.

This general pattern of no response, majority type B response and then predominantly C and D responses with increasing concentration is shared by the other two amino-acids tested, L-orn and glycine. With L-orn appeared to be more potent whereas glycine was less potent.

The concentration range for L-orn producing a response was lower i.e. 50 mM. A majority of type B responses were also seen at 100 mM of L-orn whilst at increasing concentration this pattern shift to a majority of type C and D responses.

Glycine was shown to be an experimentally negative control (28), however it did evoke changes in [Ca^{2+}]_{i} during this study. The threshold concentration for responses seen with glycine was 200 mM. Interestingly even at the highest concentration tested (500 mM) the vast majority of responses evoked by glycine were of type B. During these series of experiments glycine did not evoke any of the irreversible, type D, responses.

Mannitol, as previously described, was used as an osmotic control. Fig. 7 shows that it did however induce changes in [Ca^{2+}]_{i}. However the pattern of response were quite different to those seen with the amino acids. No responses were seen below 300 mM and the vast majority of all responses seen were of type C and D.
Figure 7. Represents the results for the categorical analysis of the calcium responses seen with L-arg, L-orn, Glycine and Mannitol. The graphs illustrate the % of cells displaying responses A, B, C or D at varying concentrations of reagent. In general for all reagents tested there appeared to be a concentration dependent shift in the % of cells that exhibited responses A-D with an increase in the most severe responses at the higher concentrations.
2) **Mean maximum response (MMR)** – The mean value for the maximum increase in [Ca\(^{2+}\)]\(_i\), (340/380 ratio) achieved when the cells are exposed to a reagent at a particular concentration (see fig 6.) The MMR can be used to compare maximum responses for a given concentration and its dose dependent variation. When plotted on a graph this can be used to compare the dose response relationships for the various reagents (fig 8).

3) **Area under the curve** – The area under the curve (340/380 ratio vs. time) was measured for a given amount of time (400 seconds in this case) for all concentrations tested (fig 8). This reflects the total Ca\(^{2+}\) entering the cytosol over a given period of time and reflects the net effect of Ca\(^{2+}\) release, entry and reuptake of Ca\(^{2+}\). This is particularly useful information in addition to the maximum response values as it is affected by the time taken to respond as well as accounting for a prolonged rise in [Ca\(^{2+}\)]\(_i\).
Figure 8. Graph a represents a comparison of the mean maximum response values for the various reagents against their respective concentrations (mM). The individual maximum responses curves for each reagent are also shown (MMR= Mean Maximum Response value for the 340/380 nm Ratio). Graph b represents a comparison of the graphs produced by plotting the mean area under the curve values (AUC) for each reagent, measured over a 400 second time period, plotted against their respective concentration (mM). Error bars represent standard error of the mean for each value.

L-arg: 100 mM [MMR=0.85, SD=0.74], n=5, cells=42. 200 mM [MMR=1.256, SD=0.12] n=5, cells=35. 500 mM [MMR=1.649, SD=0.11] n=5, cells=32.

L-orn: 50 mM [MMR=0.92, SD=0.06] n=5, cells=22. 100 mM [MMR=1.22; SD=0.89] n=5, cells=15. 300 mM [MMR=1.303 SD=0.17] n=5, cells=36.

Glycine: 200 mM [MMR=0.58, SD=0.88] n=5,cells=12. 300 mM [MMR=0.98 SD=0.06] n=4,cells=24, 500 mM [MMR=1.2, SD=0.24] n=5, cells=18.

Mannitol: 300 mM [MMR=0.801, SD=0.07] n=5,cells=20. 500 mM [MMR=1.145, SD=0.12] n=5, cells=16.
The MMR response for L-arg shows a steep initial rise at 100 mM that eventually reaches a plateau at 400 mM above which there is no corresponding increase in response with increasing concentration. However the shape of the corresponding area under the curve (AUC) graph shows a continual increase as the concentration rises. If we compare these findings with categorisation data in figure 7, we can see that at the higher concentrations there is a change in the type of response seen. The responses change from being predominantly B to C and D. Type C shows a more gradual return to the baseline whilst D represents an irreversible increase in $[Ca^{2+}]_i$. The AUC corresponds to the increase in $[Ca^{2+}]_i$ for a particular unit of time, therefore the factors affecting the value will be the size of the response and shape of the response curve. For the same MMR, response C will have a greater value than the narrow spike-like type B and response D will have the greatest value.

L-orn displays a similar MMR and AUC graph to L-arg however it is shifted to the left suggesting that L-orn evokes similar responses at lower concentrations.

Allowing for the error margins both glycine and mannitol share a similar MMR response curve however the AUC graph is different. Glycine exhibits little change in the AUC with increasing concentration however for mannitol, at the same concentration, the AUC value is greater and it continues to climb. This can be explained by considering the categorisation data in fig 7. Glycine produces predominantly type B responses throughout the experiments and therefore a relatively small AUC value whereas mannitol evokes mostly type C and D responses.

It is highly likely that some of the amino acid-evoked Ca$^{2+}$ responses at very high concentrations (>100 mM) were due to hyperosmotic effects. For the purpose of exploring these potential effects, isosmotic solutions of amino acids were used. This involved using HEPES-PSS in which 100 mM of NaCl were replaced with 100 mM
of test reagent. In addition an isosmotic control NMDG was used, (100mM NaCl replaced with 100mM of NMDG) to account for the effects of NaCl depletion.

![Graph showing proportions of responses](image)

Figure 9. A graphic representation of the proportions of responses seen with isosmotic L-arg and L-orn (HEPES-PSS in which 100mmol of NaCl have been replaced with L-arg / L-orn and as a control to account for the Na deficiency HEPES-PSS in which the 100 mM of NaCl have been replaced by the inert NMDG).

Isosmotic L-arg produced “spike-like” responses (type B) in 68% +/- 7.9% of cells whereas no responses were seen with NMDG (fig 9). All of the positive responses seen with L-arg were type B only. L-orn produced responses in 91% +/- 5.4% of which 8% displayed slow recovery responses (type c).

Effects of amino acids on CCK evoked oscillations

Physiological doses of CCK induce characteristic spike-like, oscillatory changes in cytosolic calcium. These series of experiments involved analysing the effects of L-arg and L-orn on this physiological CCK response. Isosmotic solutions of 100 mM L-arg,
L-orn and NMDG were added to viable clusters of pancreatic acini during a train of robust CCK evoked $[\text{Ca}^{2+}]_i$ oscillations.

Changes in the amplitude, frequency and area under the curve were recorded and analysed.

CCK induces its effect through an oscillatory signal of which the frequency can be used to represent the strength of the stimulus. The amplitude of the response represents the magnitude of the stimulatory signal inducing an increase in calcium release. The total amount of cytosolic calcium change in a cell for a given period of time can be represented by the area under the curve (fig 10.)

![Figure 10. A typical train of CCK evoked oscillations in $[\text{Ca}^{2+}]_i$, highlighting the variable parameters of this response that were measured.](image)

Each set of experiments included one with CCK alone, this was used as a time matched control for comparison. The experiments included a CCK only control period, (T1), the test reagent and CCK period, (T2) and the recovery period of CCK alone, (T3) (figure 11).
Figure 11. This CCK evoked response tracing shows the three distinct recorded periods: T1 (the CCK alone control period), T2 (the reagent added test period) and T3 (the CCK alone recovery period).

The reagents tested in these experiments were L-arg, L-orn and N-methyl-D-glucamine (NMDG). As previously discussed hyperosmolar solutions have implications on cell volume and ionic composition. To counter this only the isosmotic solutions of the reagents (previously described) were used.

Statistical significance was determined using Wilcoxon’s signed rank test and the normalised data within each experiment was compared (paired analysis) using a single sample t test (analysing if T2/T1 or T3/T1 is significantly different from 100%). Each parameter in the reagents experiments was compared to the corresponding parameter of the time matched control experiments using the Mann Whitney U Test and a 2-way ANOVA test of variance (unpaired analysis). The time matched control data and a summary of the analytical techniques are displayed below (figure 12).
Figure 12. Fura-2 loaded pancreatic acinar cells were stimulated with 50pM CCK to evoke calcium oscillations. T1=control time period (i.e. only 50pM CCK applied). T2=test period (i.e. 50pM CCK + isosmotic solution of reagent). T3=recovery period (i.e. 50pM CCK only). Normalisation of the data was achieved by expressing the test and recovery period parameters as a percentage of the control response – i.e. Test = T2/T1 x 100, Recovery = T3/T1 x 100. The parameters used to compare the data in each time period were frequency of oscillations, mean amplitude of the calcium peaks and the area under the curve.

There was a visible disparity between the frequency of oscillations between the test and recovery periods during the control. This does bring doubt into how reliable the frequency data is. This will, however, be discussed later.

Graphs representing the results for L-arg, L-orn and NMDG are shown below (fig 13.)
Statistically significant

Figure 13. Isosmotic reagent solutions were added to fura-2 loaded acinar cells displaying CCK evoked oscillations. Data was obtained on the effects of the reagents on frequency of oscillations, amplitude of responses and area under the curve of the responses, during the test period as well as the recovery period. The data was normalised by expressing the test and recovery
period as a percentage of the initial control period. Paired and unpaired statistical analysis was carried out using a single sample t-test, Mann Whitney U-test and a 2 way ANOVA.

The data shows that L-arg did not have any effect on the frequency of oscillations, the mean amplitude or the area under the curve. There was a decrease in the frequency of oscillations during the recovery period, which was not significant however (p=0.0631).

The data shows that L-orn increased the frequency of CCK evoked calcium oscillations as well as increasing the area under the curve during the test period. The statistical analysis showed that there was a significant increase of 30% ± 20% (p=0.0405, n=4) in the frequency of oscillations during the test period and an increase of 55% ± 20% (p=0.0426, n=6) in the frequency of oscillations during the recovery period. Additionally, there was a significant increase of 7.12% ± 4.5% (p=0.0001, n=4) in the area under the curve during the test period. This may indicate an increase in calcium entering the cytosol during this period.

In order to maintain a control for replacing 100 mM of sodium in the isosmotic solutions, 100 mM NMDG was also used as a test reagent. Interestingly the data shows that during the experiments with NMDG; the frequency of CCK evoked calcium oscillations were reduced. The statistical analysis shows that there was a significant decrease of 63% ± 7% (p=0.006, n=4) in the frequency of CCK evoked calcium oscillations during the test period with 100mM NMDG. This suggests that reducing Na has a detrimental effect on [Ca^{2+}]_{os} oscillations and may also underestimate any stimulatory effect of L-arg or L-orn. However, the result with the inert control, NMDG, further questions the reliability of frequency data on interpreting the response.
Effects of amino acid on cell death

Establishing whether L-arg and L-orn induce acinar cell death is an important baseline in the process of investigating the cellular effects in this experimental model of inducing AP. Using the haemocytometer enabled us to determine the total number of cells in the test field. Trypan blue staining identified those damaged or dead cells. The % of dead cells was measured at 30, 60, 120 and 180 mins. from introduction of reagent, and compared to a time matched control and the experimentally negative control glycine.

*Statistical significance (p<0.05)

Figure 14 – Trypan blue exclusion experiments ; to quantify the effects of L-arg and L-orn on cell death and compared to the control Glycine and a time matched control. 100 mM isosmotic L-arg n=5, L-orn n=6 and Glycine n=5, measuring the % dead cells counted using a haemocytometer. This was done at 0, 30, 60, 120 and 180 mins. Error bars represent SEM. Statistical significance was determined using 2-way ANOVA.

The data reveals both L-arg and L-orn cause a profound increase in cell death as compared to glycine. Throughout the test period glycine did not cause any significant
increase in cell death as compared to the time-matched control. By 30 mins. L-orn induced a greater percentage of cell death as compared to L-arg (57% +/- 8.7% and 36% +/- 4.2% respectively)

Oxidative Stress Experiments
This study also aimed to determine the mechanism by which isosmotic L-arg and L-orn exert their toxic effects on pancreatic acinar cells. One of the proposed mechanisms is through induction of oxidative stress through the generation of free radicals such as peroxynitrite. To investigate this further, fluorescent DCF dye was used to detect and measure oxidative stress on the pancreatic acinar cells. The impact of adding isosmotic solutions of 100 mM L-arg or 100 mM L-orn were compared to the maximal response generated by the positive control, 3 mM H_2O_2. This concentration of H_2O_2 has been shown to exhibit rapid and robust increases in DCF fluorescence which over prolonged periods (20-40 mins) causes cell lysis (39).

In preliminary control experiments, some cells exhibited an upward drift in DCF fluorescence trace, this was prior to the addition of any test reagent or the positive control. This may have been due to an endogenous increase in oxidative stress. It may also have been due to phototoxicity. To account for this effect it was necessary to identify the slight drift in fluorescence during the control period. This baseline could then be subtracted from any observed change in fluorescence with each test reagent. This allowed us to isolate and calculate the increase in fluorescence due to the test reagent alone.

The values obtained after subtraction of the baseline trace, were used for the statistical analysis. It allowed us to identify the true response induced by the reagent as well as the response seen with the control, 3mM H_2O_2. The maximal increase in fluorescence
for both the reagent and H$_2$O$_2$ were recorded. The reagent response was then expressed as a percentage of the maximal increase in fluorescence seen with H$_2$O$_2$.

Multiple responses for L-arg and L-orn were obtained and normalised to the maximal H$_2$O$_2$ response (100%). A single sample t test was used to determine whether the maximal increase in fluorescence as a result of the reagent was significantly different from 0 (fig 15.)

**L-arg**

![Graph of L-arginine response](image1)

**L-orn**

![Graph of L-ornithine response](image2)

Figure 15. The effects of amino acids on oxidative stress; the response curves for the changes in DCF fluorescence against time when L-arg and L-orn are added. These responses were compared to the maximal response seen with 3 mM H$_2$O$_2$. 
The maximal change in fluorescence detected when L-arg and L-orn were added was expressed as a percentage of the maximal change in H₂O₂ (3 mM). This gave relative oxidative stress values for the reagents. The data was analysed using a single sample t test.

L-arg produced a significant oxidative stress response of 21.7% ± 4.5% (p=0.0002, n=18). L-orn also produced a significant oxidative stress response of 36.8% ± 5.7% (p=0.0006, n=7).

* Statistically significant

Figure 16. 100mM L-arg and 100mM L-orn generate a significant oxidative stress response in pancreatic acinar cells as compared to 3 mM H₂O₂.

**DAF-FM Experiments**

The aim of this series of experiments was to determine whether L-arg and L-orn induced changes in intracellular NO. The reagents used were isosmotic solutions of 100 mM L-arg and L-orn and compared to the response seen with potent NO donors. However, unfortunately neither of the +ve control reagents (SNAP, sodium nitroprusside and GTN) were able to induce a significant change in DAF-FM fluorescence. This made interpretation of these data impossible. Either the dye was not effective, there was in fact no increase in NO or the increase in NO was too short-lived.
Discussion

Acute pancreatitis continues to contribute to mortality and morbidity figures on a global scale. Despite significant advances in our understanding of the course of the clinical disease, little has changed with regards to disease modulating treatment. The general consensus is that the gap in our understanding is at a cellular level and in particular, the triggering mechanism. To aid the exploration of physiological and cellular theories numerous experimental models have been described. As previously discussed there are limitations with all of these models. The L-arg model is easily reproducible and produces a predictable dose dependent pathology. The main criticism of it being that it does not represent a human aetiology for AP. However, this model accurately reproduces the progression of the human disease and has proved useful. There is therefore a clear rationale for understanding the molecular mechanisms for L-arg induced AP.

The main aim of this study is to provide more information on the intracellular changes induced by L-arg and whether these changes can be linked to pathophysiology of the clinical disease that is acute pancreatitis.

The overarching hypothesis of this project is that L-arg (and the related L-orn) activate a CaSR-like, amino acid sensing GPCR (such as GPRC6a) leading to cytotoxic calcium overload and the characteristic cell death which are hallmarks of AP. If this is proved to be true, then L-arg induced experimental AP shares similarities to the major aetiologies of the human disease (e.g. bile acids, ethanol metabolites) in which cytotoxic calcium overload is a common feature.
Calcium signalling

The associated rise in calcium preceding the normal zymogen exocytosis is well established (41). There is also a well documented association between a prolonged rise in acini $[Ca^{2+}]_i$ and the premature intracellular activation of trypsinogen that leads to glandular autodigestion in AP (13). Cytosolic calcium overload is also cytotoxic, inducing both apoptotic and necrotic cell death (5).

The physiological changes in calcium, such as those seen with agonists such as CCK are described as spike like (type B) in which $Ca^{2+}$ is rapidly released from the ER and rapidly cleared from the cytosol. These were seen readily with physiological CCK as well as with L-arg, L-orn and Glycine but not with mannitol. This suggests that the amino acids do induce calcium signals in acinar cells that mimic the physiological responses. This spike-like change returning to the resting baseline is reminiscent of a G protein receptor mediated stimulus. The data reveals that both L-arg and L-orn have a threshold concentration (100 mM and 50 mM respectively), below which there is no response and when reached there is a rapid and significant response. This is consistent with the “all or nothing” type receptor mediated $Ca^{2+}$ responses. The initial responses seen with both L-arg and L-orn were spike-like (type B.) These rapid, spike like increases in $[Ca^{2+}]_i$ are reminiscent of the fast IP3 mediated $Ca^{2+}$ release responses induced by activation of a GPCR, such as the CaSR or the amino acid sensing GPRC6a receptor. These responses alone however, are unlikely to be significant in the disease process of AP. This is partly because the type B responses were also induced by the experimentally negative control glycine. Glycine was chosen as a negative control because it has been used as a negative experimental control in L-arg and L-orn induced AP (28). However glycine has been shown to activate GPRC6a (19). As previously discussed it is the calcium overload response
that is of more relevance in the disease process. This is better represented by responses C and D (fig 17) in which Ca\(^{2+}\) either slowly recovers or is irreversibly elevated.

![Figure 17 Categorisation of calcium responses as previously described.](image)

Interestingly, as the concentration of L-arg and L-orn increases, these slowly recovering and cytotoxic responses (C and D) are the predominant responses seen and are reported to induce apoptotic and necrotic cell death (5). At concentrations above 300 mM both L-arg and L-orn display a shift towards producing persistently elevated levels of cytosolic calcium.

The concentrations of reagents used to induce the disease experimentally in animals ranged from 1-2M (18,27,28). Due to the effects of peritoneal cavity dilution and fluid sequestration, induced by a chemical peritonitis, the concentrations of the amino acids the pancreas will be exposed to will be less. It is difficult to ascertain the exact concentrations the gland is exposed to however it is not unreasonable to estimate they are likely to be greater than the concentrations (0.3-0.5 M) used in this study to induce calcium overload, particularly in the earliest stages. Also the results show that glycine, an experimentally negative control (28), did not induce the irreversible rise in [Ca\(^{2+}\)]\(_i\) (type D) responses even at higher concentrations thus further supporting the experimental findings. However irreversible changes (type D responses) were seen at
the highest concentrations (500 mM) with the osmotic control mannitol. This was used as an inert sugar solution and unable to enter the cell. The only comparable feature was its concentration therefore implying the result was due to an osmotic effect on the cell. Although mannitol has not been tested clinically it is unlikely to induce AP.

These responses may well represent abnormal changes in calcium, however at the higher concentrations, they are indistinguishable from those seen with the control. It is difficult, therefore, to isolate amino acid induced responses from those that are a consequence of extremely hypertonic solutions. Osmotic effects will lead to a decrease in cell volume causing relative changes in $[\text{Ca}^{2+}]_i$ and movement of $\text{H}^+$ ions will alter cell pH. Changes in cell volume and pH, if significant enough, can have an impact on cytosolic composition affecting intracellular calcium signalling. These potential osmotic effects, although unlikely to be responsible for causing the pathological changes seen in AP, do not completely explain the range of responses seen. In particular the spike-like (type B) responses, which were seen at the lower concentrations only with the amino acids. The mechanisms of inducing these responses may still be mediated by a G-protein receptor complex, however are unlikely to be solely responsible for initiating AP.

To further investigate the potential osmotic effects, isosmotic solutions of 100mM L-arg and L-orn were used. Despite removing the added osmotic gradient it did not abolish the responses seen with L-arg and L-orn whereas no responses were seen with the control, NMDG. Isosmotic solutions required replacing sodium and chloride ions; this depletion of $\text{Na}^+$ and $\text{Cl}^-$ can have an impact on $\text{Na}^+$ dependent cellular mechanisms. However this is unlikely to explain the results as no responses were seen
with the control. This further information reinforces the fact that L-arg and L-orn induce changes in intracellular calcium independent of the osmotic effects.

As previously discussed, there is well-documented evidence for L-amino acid activation of GPRC6a (table 1) (19).

This data supports the potency of the different amino acids in their ability to activate the receptor complex, with L-orn being the most potent and glycine the least of those tested. However there is one major caveat to this, the unit of concentration is μM. The concentrations used in animal models as well as this study, however, are in mM. This several-fold order of magnitude difference constitutes an important factor when interpreting data. It has already been established that the concentration of L-arg that the pancreas will be exposed to experimentally will be lower. This dilution effect in a rodent abdomen, however, is unlikely to be of this order of magnitude. This evidence suggests that if responses seen were solely attributed to activation of GPRC6a they would have been seen at much lower concentrations. It is important to note that most receptor activation data is achieved on isolated GPRC6a often in an over-expressed system, which is likely to differ from the results seen in the experimental models.

The circulating gastrointestinal peptide CCK is of particular interest to this study. In normal acinar cell physiology it is known to activate exocytosis of zymogens through changes in cytosolic calcium. It mediates its effect through IP3 production as a result of activation of a G-protein receptor complex (CCK-1 receptor.) Furthermore its analogue caerulein, in excess, is known to induce AP. The mainstay of theories for CCK induced AP are centred on hyperstimulation and premature activation of proteolytic enzymes (23). The mechanism is likely to be inappropriate enzyme activation as a result of cytosolic calcium overload. This particular aspect of cellular physiology is pertinent to this study. L-arg and L-orn are known to act as ligands for
the CaSR-like GPRC6a but not known to interact with CCK receptors. The effects of both L-arg and L-orn on the CCK-evoked responses have not previously been described.

As previously discussed, analysis of the data revealed three different measurable characteristics of the CCK response: the frequency, amplitude and area under the curve.

L-arg had no significant effects on the CCK evoked responses. L-orn, however caused an increase in both frequency and area under the curve of the CCK response. The frequency of the response represents the size of the stimulus. Area under the curve reflects the amount of calcium moving in and out of the cytosol for a particular period of time and is affected by both frequency and amplitude. The suggestion that L-orn increases the strength of the response and the cytosolic concentration of calcium may imply L-orn potentiates the CCK evoked response. The significance of this however is questionable in relation to the disease process. There is no evidence of calcium overload, which would be necessary to link this to experimental findings. This may well change with greater concentrations of reagent however exceeding 100 mM isosmotic solutions would require replacing all the Na in solution. As previously discussed the repercussions of doing this would be disruption of Na dependent ion exchange causing numerous other effects such as changes in cell volume and pH. The other main caveat to the significance of this result is that NMDG also induced significant changes in the frequency of the CCK response. This was used as a control for sodium depletion but that itself is unlikely to explain the results seen as isosmotic L-arg had no effect. Care must be taken in interpreting the data with regards to frequency. The frequency of oscillations is not a continuous parameter and the data is binary coded, where a small change in the raw data can be translated as a large change.
in the normalised data (i.e. an increase of 4 spikes, per time period, to 6 translates as an increase in 50%). Additionally, there may be a latency period between applying CCK and initiation of CCK evoked Ca\(^{2+}\) oscillations. This tends to happen during the initial control period only; this will skew analysis of the frequency data. Therefore, the area under the curve data may be the most reliable source of information as it incorporates both frequency and amplitude and can thus reflect changes in both those parameters.

In conclusion the data provides no substantive evidence that isosmotic solutions of 100 mM L-arg or L-orn impair the physiological CCK evoked responses in a way that is likely to be significant in the disease process.

Cell death assays reveal that both isosmotic 100 mM L-arg and L-orn induce significant cell death over a two hour period as compared to a time matched control. This was not seen with glycine, which showed no significant difference to the time matched control. These data are basically in line with the experimental model described by Rakonczay et al (28). Furthermore L-orn was also shown to be more cytotoxic for the same concentration as L-arg. This further challenges the NO dogma that NO production is responsible for cytotoxicity and cellular injury during this experimental model of AP. This series of experiments also challenge the previous notion of osmotic effects being the major contributor to cell injury.

The results, however, do not support the theory of cellular injury being mediated solely by a receptor such as the CaSR or GPRC6a. The reason for this being that 100 mM L-arg and L-orn both produce predominantly spike like, reversible changes in [Ca\(^{2+}\)], only rather than Ca\(^{2+}\) overload.
To further investigate potential cytotoxic mechanisms a further series of experiments were carried out to investigate changes in intracellular oxidative stress induced by these amino acids.

Cellular injury via the formation of intracellular oxygen free radicals has been the focus of many theories on AP. The data in this study shows clearly that both L-arg and L-orn induce a significant cellular oxidative stress response as compared to 3 mM H$_2$O$_2$ which induces a maximum increase in oxidative stress (39). The maximal responses seen with L-arg and L-orn were expressed as a percentage of this positive control response and therefore, the two are paired. It stands to reason that a less robust H$_2$O$_2$ response would produce results that would increase the apparent magnitude of the test reagent response (as it would be expressed as percentage of a smaller value). Therefore, the experimental values of the test reagent responses are dependent upon the concentration of H$_2$O$_2$ used. The rational for the dose used was that 3mM has been shown to give a robust oxidative stress response (39) without being cytotoxic. A particularly interesting result was seen with L-orn. This NO independent metabolite of L-arg was even more potent at inducing oxidative stress than L-arg. In view of the theories centred on NO and the formation of RNS and ROS, L-orn would not be expected to have shown such a results. This further challenges the idea that the formation of cytotoxic free radicals are as a response to NO production and re-enforces the need to understand the mechanisms of this particular amino acid’s ability to induce AP. In reference to the overall aims laid out in this study, the DCF data confirms that L-arg does induce a degree of oxidative stress, which has cytotoxic potential. This may well be due to its NO producing potential, however the results with L-orn suggest an alternative mechanism may be present. Both these amino acids
can induce changes in intracellular calcium but there is currently no clear link between changes in cytosolic calcium and the generation of intracellular ROS.

Reviewing the literature has raised numerous issues that must be explored in an attempt to further our understanding in the initial and arguably most critical steps in AP. Being able to modulate this potentially catastrophic disease process has continued to evade the medical profession. The difficulty in developing a significant treatment for this disease is in part because clinical detection often occurs late, i.e. once the pathological process is already well established. The mainstay of management is limiting the consequences of a widespread, systemic inflammatory response and not at modulating the triggering sequences at a glandular level. For these reasons, this body of work focused at improving our understanding of the earliest stages of the AP. There has been much controversy about the choice of an experimental model for AP to study the disease. As previously discussed the reasons for using L-arg induced AP as a focus was because of its well-established history as a model that produces dose dependent acinar cell necrosis, which mimics the severe human disease.

There has been a lot of interest in NO and oxygen free radicals but there is discrepancy in experimental data as well as clinical trials using anti-oxidant therapy and to date have not provided definitive information on the triggering factors in AP.

The aim of this body of work was to test alternative theories to explain the earliest cellular stages in this experimental model of AP.

Fig 18 represents a summary of theories tested in this body of work and by discussing the findings we will look to see if the results in this study provide further information in understanding the cellular mechanisms involved in L-arg induced experimental AP.
Figure 18. Theories for L-arginine induced acinar injury.

A. Represents the theory that calcium overload is induced by L-arg stimulation of a CaSR like GPRC6a through release of IP3.

B. Represents intracellular metabolism of L-arg to NO, which in excess can combine with superoxides to form the highly cytotoxic peroxynitrite (ONOO−).

The calcium imaging experiments did confirm that both L-arg and L-orn could induce changes in cytosolic calcium that would be consistent with a receptor mediated response. At the lower concentrations tested this spike like rapid response is unlikely in itself to be pathological. However at the higher concentrations that induced calcium overload, it is difficult experimentally to separate the responses from those seen with hypertonic control solutions.

In conclusion neither the presence nor absence of a CASR or GPRC6a like receptor can be confirmed. The main argument against this specific theory is that no responses were evoked by either L-arg or L-orn at doses that would have been adequate to stimulate GPRC6a. The EC50 values for the L-amino acid activation of this receptor
are in the µM range. Although the amino acids could be activating a CaSR with much lower affinity.

The results seen with L-orn are very significant. The formation and metabolism of L-orn is independent of NO. This amino acid, however, is more potent experimentally at inducing AP (28). It produces robust changes in calcium at lower concentrations than L-arg, induces more oxidative stress as well as cell death. This information challenges many current theories as well as providing an important focus for future work.

This study has shown that L-arg induces changes in intracellular calcium, oxidative stress and cell death. It has also shown the NO independent metabolite, L-orn, is more potent than L-arg in inducing these effects. These effects may well be linked by a universal mechanism and maybe significant in the early phase of AP. However with the high concentrations used experimentally it is difficult to isolate them from numerous other physiological processes occurring.
Further Work

1) Experimentally the mode of administration of L-arg is through intra-peritoneal injection. The concentrations range from 1-2 M which result in infusion of a very hypertonic solution into the abdominal cavity. This will have significant osmotic effects. Fluid sequestration from the visceral organs will cause dilution of the reagent. This will be further exacerbated over time as a result of oedema from a chemical peritonitis. This factor of dilution has not been well documented in the literature. The actual concentration the pancreas is exposed to is clearly important when designing experiments at a cellular level. Without actual peritoneal sampling in animals the value will always be an approximation however calculations based on peritoneal dilution and reviewing this work in other models may be useful.

It may be also important to consider the effects of the intraperitoneal injections upon other organs and the ramifications on the systemic inflammatory response in AP.

2) The current theories on L-arg induced AP do not extend to explaining the effects that L-orn has on both isolated pancreatic acinar cells as well the gland as whole. The literature suggests that L-orn produces a more severe pancreatitis experimentally (Rakonczay). This study has shown L-orn to be more potent at inducing changes in intracellular calcium and oxidative stress. This raises a need to improve our understanding of the pathobiology of L-orn induced AP in an attempt to link the demonstrated physiologic responses to the disease process.
3) Clearly differences in osmolarity will have physical effects on the cell with regards to movement of water and ions however this needs to be quantified by carrying out measurements of changes in cell volume and pH. Also to explore the impact of changes in cytosolic calcium as a result of rapid changes in both cell volume and pH.

4) Though the presence of a cell receptor can be demonstrated through its functional analysis a direct comparison with a model where the receptor is absent strengthens the evidence. Further studies can be designed to repeat the experiments with GPRC6a “knockout” animals. A comparison of the amino acid induced changes in intracellular calcium in isolated acinar cells from rodents in which GPRC6a is not expressed with those seen in this study will provide further evidence on the role of this receptor in cellular calcium signalling.

5) As clearly demonstrated by a review of the literature the role of NO is unclear. It has been an area of significant interest in both experimental and clinical pancreatitis. Its effects have been proposed to be both beneficial and detrimental in the disease process. Clearly there is a need to further understand the effects of NO at a cellular level and in particular on the changes in intracellular calcium. Robust monitoring of the changes in intracellular NO in isolated acinar cells requires specialised techniques. In particular the loading of cells with NO specific dyes and identification of an appropriate control. The changes in intracellular NO, as well as other
nitrosative and oxygen species, induced by L-arg (and L-orn) has proved to be difficult however is an important series of experiments to pursue.

6) Exploring alternative theories on the mode in which L-arg/L-orn induces cell death and AP. Cellular homeostasis is dependent on numerous physiological functions. It is possible that these amino acids may induce other effects such as mitochondrial dysfunction. Further experiments can be carried out to be specifically measure changes in: ATP, mitochondrial membrane potential and NADH.
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